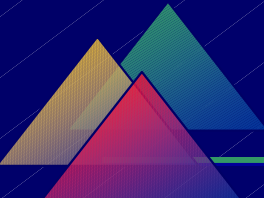


# Arsenic Speciation Methods for Studying the Environmental Fate of Organoarsenic Animal-Feed Additives

Three overlapping triangles in shades of blue, green, and red, positioned to the left of a horizontal line that spans the width of the slide.

Georges-Marie Momplaisir, Charlita G. Rosal,  
and Edward M. Heithmar

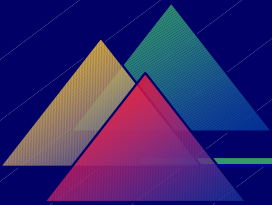
U.S. Environmental Protection Agency  
Office of Research and Development  
National Exposure Research Laboratory  
P.O. Box 93478  
Las Vegas, NV 89193-3478

# To cite this presentation:

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Momplaisir, G.M; C.G. Rosal; E.M. Heithmar “Arsenic Speciation Methods for Studying the Environmental Fate of Organoarsenic Animal-Feed Additives,” U.S. EPA, NERL-Las Vegas, 2001; (TIM No. 01-11) available at:

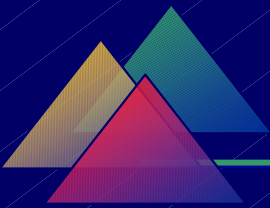
<http://www.epa.gov/nerlesd1/chemistry/labmonitor/labresearch.htm>



# Notice

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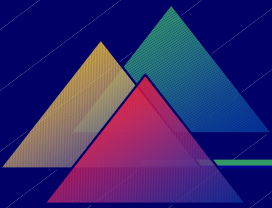
- The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), funded this research and approved the materials that formed the basis for this presentation. The actual presentation has not been peer reviewed by EPA.
- This research project is a collaboration between the U.S. Geological Survey (USGS) and the U.S. EPA. Analytical data presented here do not include work performed at USGS laboratories.
- These results are only preliminary. The presentation (October 2001) will be updated to include new analytical data.



# Presentation Outline

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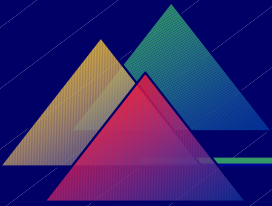
- ✓ Rationale for the study
- ✓ Goals and objectives of the study
- ✓ General background on arsenic (As) as an environmental issue
- ✓ Arsenic animal feed additives
- ✓ Facts about *roxarsone*, the most common arsenic animal feed additive
- ✓ Possible pathways for the biotransformation of roxarsone
- ✓ Toxicity values of selected arsenic compounds
- ✓ Approaches to chemical analysis of arsenic species
- ✓ HPLC separation of As species
- ✓ Separation of As species by capillary electrophoresis (CE)
- ✓ Coupling HPLC and CE with ICPMS
- ✓ Preliminary results of HPLC-ICPMS determination
- ✓ Conclusions
- ✓ References



# Rationale of the Study

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- Organic arsenic compounds are extensively added to the feed of animals (particularly poultry and swine) in the United States to improve growth rates by controlling parasitic diseases.
- The resulting arsenic-bearing wastes are currently introduced to the environment, and even used to fertilize croplands.
- Little is known about the environmental fate of these compounds.
- There has been a recent major expansion in concentrated animal feedlot operations (CAFOs). The potential risks from wastes generated by these operations must be assessed.

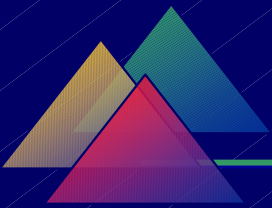


# Goals of the Study

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- Develop analytical methods to measure a number of inorganic and organic arsenic species in a variety of environmental matrices.
- Elucidate environmental transformations undergone by organoarsenic animal-feed additives and determine their environmental fate; assess the potential for substantially increased exposure of humans and aquatic organisms to arsenic.





# Specific Objectives of the Study

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- Development of analytical means to measure organoarsenic animal-feed additives and their biotransformation products, as well as common inorganic and organic arsenic species. This includes development of optimal extraction, separation, and detection approaches. More emphasis will be given in this study to roxarsone (3-nitro-4-hydroxyphenylarsonic acid), the most extensively used arsenic feed additive in the poultry industry.
- Investigation of the arsenic species present in poultry litter from poultry-house operations using roxarsone-treated feed. Temporal trends for arsenic species in the incubated litter will also be studied.
- Arsenic speciation study of soil treated with roxarsone-amended litter.
- Collection and arsenic speciation analysis of samples from fields fertilized with litter from poultry-houses using roxarsone. Samples will include litter-amended soil, as well as water, suspended sediments, and bottom sediments from streams draining the fields. Two study areas have been identified: poultry-producing areas of the Delmarva Peninsula, VA and the Neuse River Basin, NC.

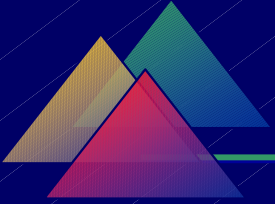


# General Background on Arsenic as an Environmental Issue

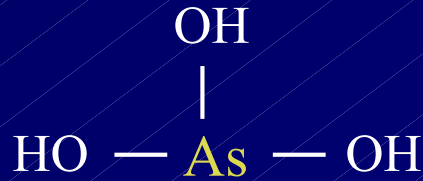
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- Arsenic species most likely to account for human exposure include:
  - inorganic arsenite and arsenate
  - methylated anionic species
  - volatile arsenic hydrides
  - organoarsenic species in food.
  
- Acute and chronic toxicity is highly species-dependent
  - inorganic arsenic
  - Methylated arsenic

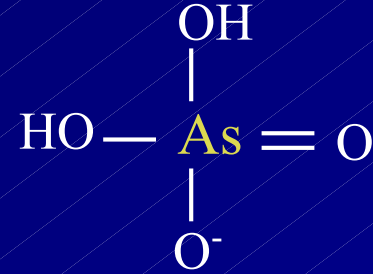




# Common Inorganic Arsenic Species



Arsenite [As(III)]



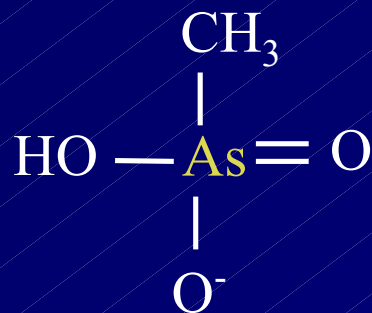
Arsenate [As(V)]

- Arsenite and arsenate are the predominant non-mineral species of arsenic in the environment.
- They are the most common mobile species in water, soil, and sediment.
- As(III) and As(V) are introduced into the environment from natural sources (e.g., volcanic activity and weathering of minerals) and from anthropogenic activity (e.g., ore smelting, burning of coal, degradation of organoarsenic pesticides).
- These compounds are highly toxic to organisms, including humans. Arsenite, being more water-soluble than arsenate, is more mobile and more available to organisms (LD<sub>50</sub> values of selected arsenic compounds are reported in Table 1).

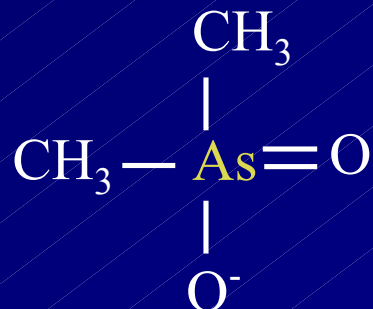


# Common Methylated Species & Volatile Hydrides of Arsenic

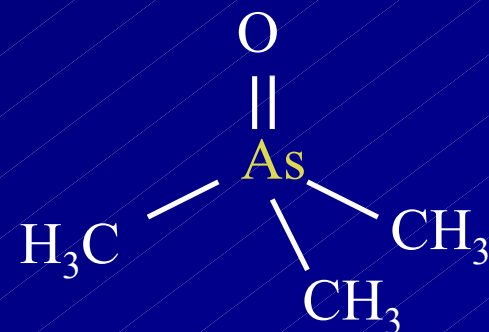
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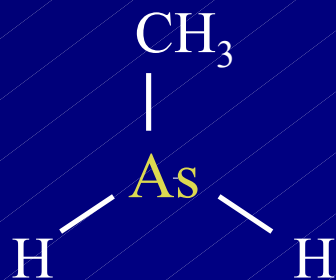
Monomethylarsonic acid  
MMA(V)



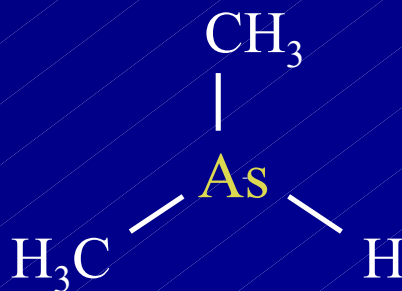
Dimethylarsinic acid  
DMA(V)



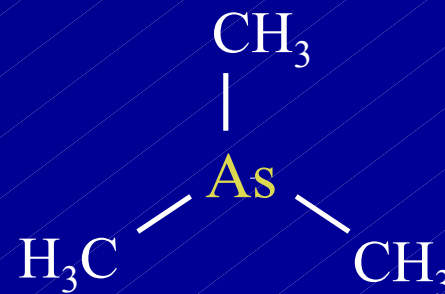
Trimethylarsine oxide  
(TMAO)



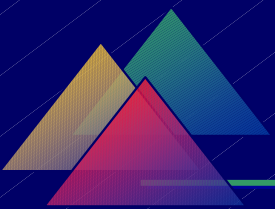
Methylarsine



Dimethylarsine



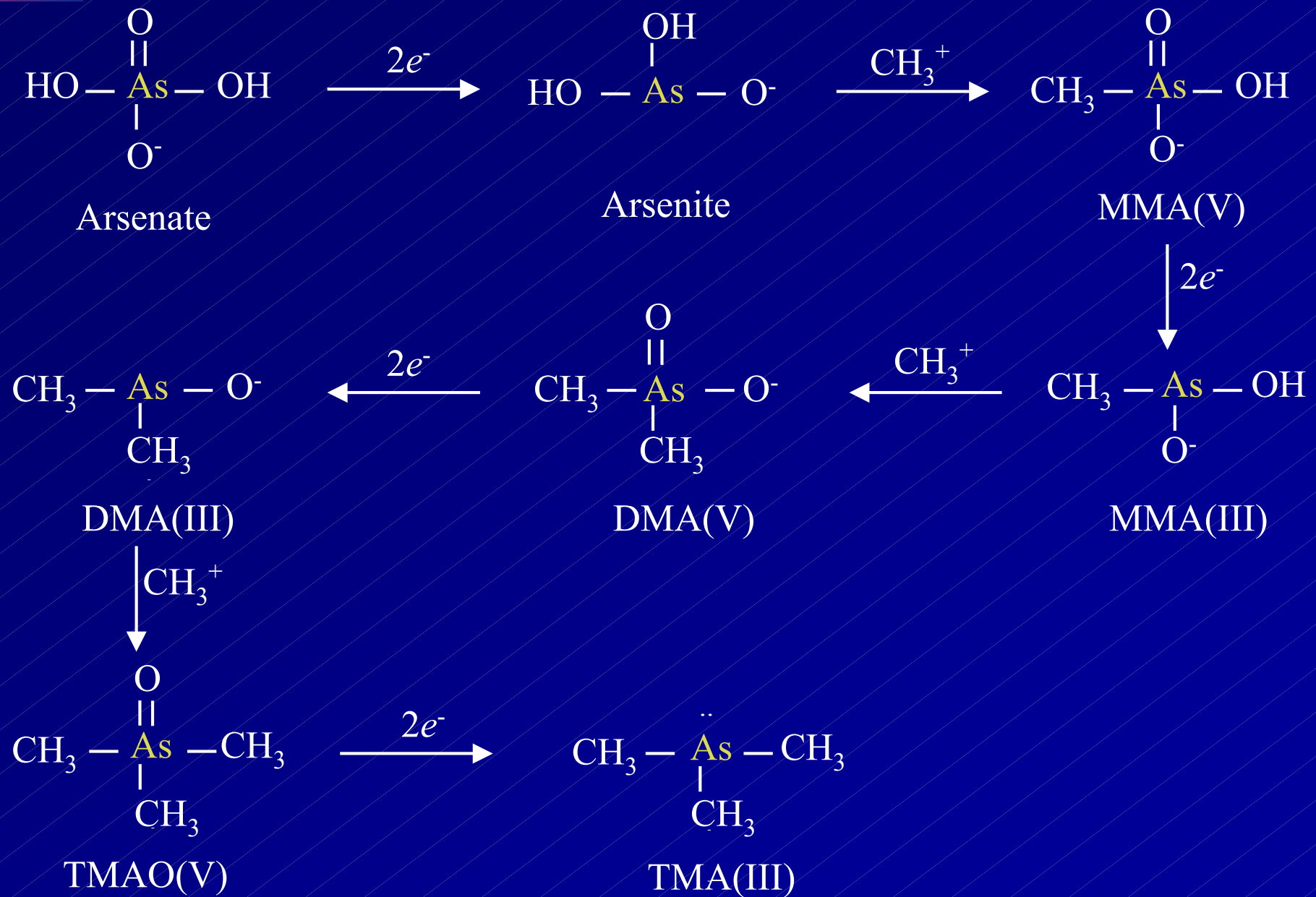
Trimethylarsine

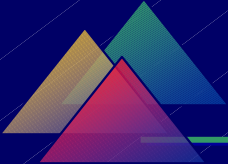


# Common Methylated Species & Volatile Hydrides of Arsenic Continued

- Presence of these compounds in environmental samples is primarily attributed to biological methylation of inorganic arsenic.<sup>1</sup>
  - Methylation of inorganic arsenic can take place in soil and sediment mediated by microorganisms, and in aquatic and terrestrial plants and animals, including humans.
  - According to Challenger,<sup>2</sup> the biomethylation of arsenic involves repeated steps of reductions, methylations, and oxidations. The proposed methylation pathway is outlined in Figure 1.
  - Recent studies indicate that the necessary intermediates monomethylarsonous acid ( $\text{MMA}^{\text{III}}$ ) and dimethylarsinous acid ( $\text{DMA}^{\text{III}}$ ) are more toxic than the inorganic arsenic species.<sup>3-6</sup>
  - Methylation in plants and animals was thought to detoxify inorganic arsenic. However, recently Le et al.<sup>7</sup> suggest that this should be reconsidered in light of the toxicity of  $\text{MMA}(\text{III})$  and  $\text{DMA}(\text{III})$  and their identification in human urine.<sup>7-9</sup>

# Figure 1. Pathway for Biomethylation of Arsenic as proposed by Challenger<sup>2</sup>





# Organoarsenic Compounds in Food

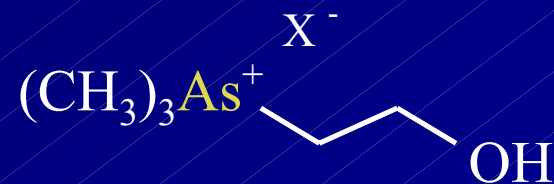
- Seafoods, especially fish and shellfish, are the most appreciable sources of dietary arsenic intake.<sup>1</sup>
  - The chemical structures of some organoarsenic compounds identified in marine organisms are shown on the next slide. The arsenosugars have been identified in various algae and phytoplankton.<sup>10-14</sup> Arsenobetaine is present in marine animals independently of their feeding habits and trophic levels.<sup>15</sup> It is the main arsenic species in the animals and can be accompanied by small amounts of arsenocholine, tetramethylarsonium ion, and other minor arsenic constituents.
  - The toxicities of arsenobetaine and arsenosugars are reported to be considerably lower than inorganic arsenicals.<sup>16-18</sup> When fed orally to mice LD<sub>50</sub> of arsenobetaine was >10 g/kg. According to Hanaoka et al.,<sup>19</sup> although arsenobetaine itself is considered as non-toxic, some degradation products formed on cooking could be toxic.
  - Biosynthesis pathways leading to the formation of arsenobetaine and some arsenosugars have been elucidated by Edmonds et al.,<sup>20</sup> and Francesconi.<sup>21</sup>



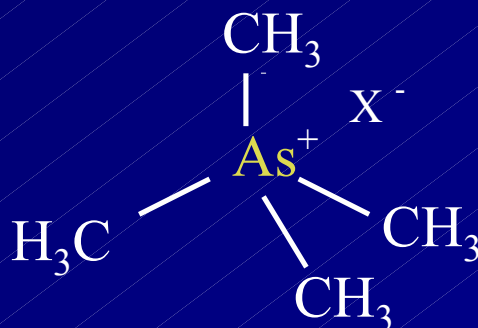
# Some Organoarsenic Compounds Identified in Seafood



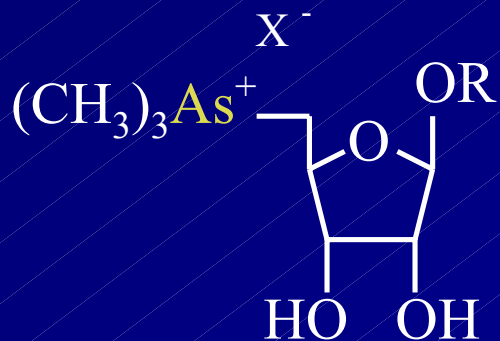
Arsenobetaine



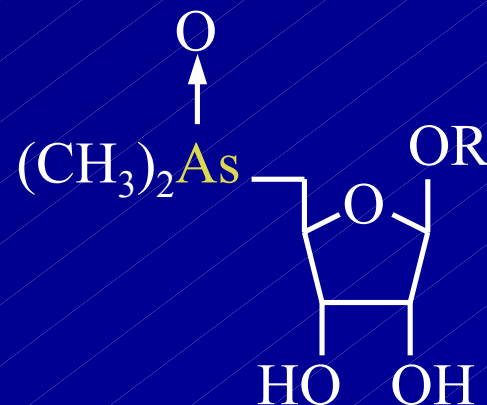
Arsenocholine



Tetramethylarsonium ion

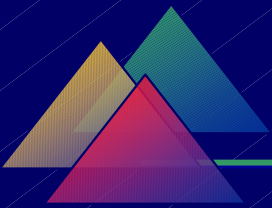


Trimethylarsonio-ribosides



Dimethylarsinoyl-ribosides





# Human Exposure to Arsenic

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## ➤ Total Diet

According to the Food and Drug Administration's (FDA) Total Diet Study, average adults consume about 53 : g arsenic per day. Approximately 20% is inorganic arsenic (10-14 : g/day).

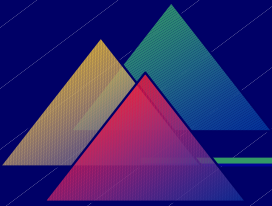
## ➤ Drinking Water

Drinking water contains mostly inorganic arsenic.

An adult drinking 2L/day of water containing 10 : g/L of arsenic would obtain approximately 20 : g/day of inorganic arsenic from drinking water alone.

## ➤ Air

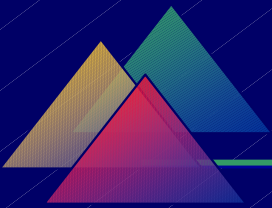
In EPA's national air databases, arsenic concentrations range from 0.003-0.03 : g/cubic meter which accounts for less than 1% of exposure.



# Arsenic Health Effects

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- Inorganic arsenic is a multi-site human carcinogen. Evidence from studies in Taiwan, Chile, and other parts of the world suggest that ingestion of arsenic at levels over 100F g/L can contribute to cancers of skin, bladder, and lung.<sup>22-32</sup>
- Non-cancer effects include gastrointestinal (GI), neurological, dermal, hematological, cardiovascular, peripheral vascular and immune system effects. Diabetes has also been linked to arsenic exposure.
- Most of the chronic arsenic poisoning cases are caused by the ingestion of inorganic arsenic from drinking well water.<sup>22-27</sup>
- A new study suggests that arsenic interferes with hormones, making it a potent endocrine disrupter.<sup>33</sup>

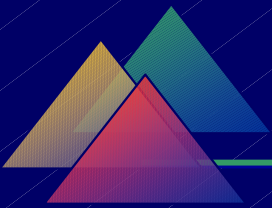


# Regulatory Status for Drinking Water

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- National Research Council concluded that the current maximum contaminant level (MCL) in drinking water of 50 : g/L is not sufficiently protective and therefore requires downward revision.<sup>22</sup>
- On January 22, 2001 EPA issued a new final arsenic standard of 10 : g/L in drinking water, with a compliance date of January 23, 2006.<sup>34</sup>
- EPA extended the effective date of the rule to February 22, 2006 (May 22, 2001 66 FR 3761) in order to conduct independent reviews of the science, the costs, and the benefits associated with the arsenic rule.<sup>35</sup>

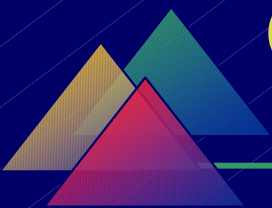
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# Regulatory Status for Drinking Water

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- In September, the National Academy of Sciences presented the National Research Council report, “Arsenic in Drinking Water: 2001 Update” [www.nap.edu/catalog10194.html](http://www.nap.edu/catalog10194.html).
- The August cost review report is available at [www.epa.gov/safewater/ars/ndwac-arsenic-report.pdf](http://www.epa.gov/safewater/ars/ndwac-arsenic-report.pdf), and the cover letter, [www.epa.gov/safewater/ars/ndwac-aug2001letter.html](http://www.epa.gov/safewater/ars/ndwac-aug2001letter.html), contains an additional recommendation.
- The August Science Advisory Board report on arsenic benefits is available at [www.epa.gov/sab/ec01008.pdf](http://www.epa.gov/sab/ec01008.pdf).
- Later this year EPA will request comment on its preliminary decision on the drinking water standard.



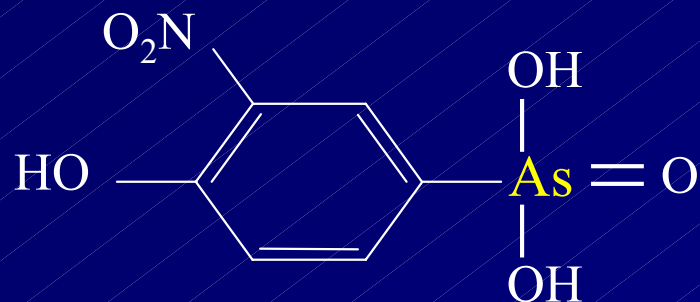
# Organoarsenic Animal-Feed Additives

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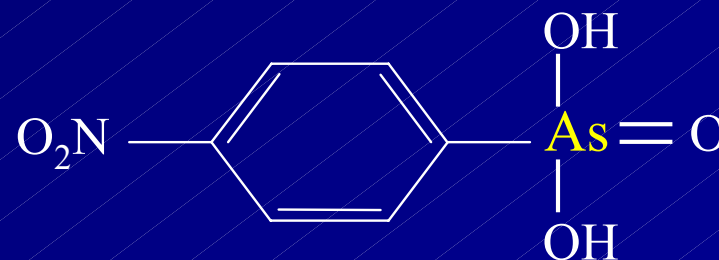
- Organoarsenic compounds commonly used as feed additives include roxarsone(3-nitro-4-hydroxyphenylarsonic acid, 3-NHPAA), p-arsanilic acid (p-ASA), p-ureidophenylarsonic acid (p-UPAA), and 4-nitrophenylarsonic acid (4-NPAA).
- Variation in the aromatic substituents results in differences in the biological activity, and therefore the FDA-approved uses of the chemicals.<sup>36</sup> Roxarsone and p-ASA are approved for controlling coccidial intestinal parasites in both poultry and swine, whereas p-UPAA and 4-NPAA are approved only for controlling blackhead disease in turkey.



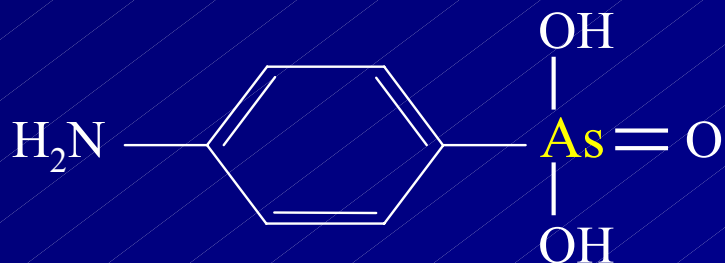
# Four Arsenic Compounds Used as Animal Feed Additives



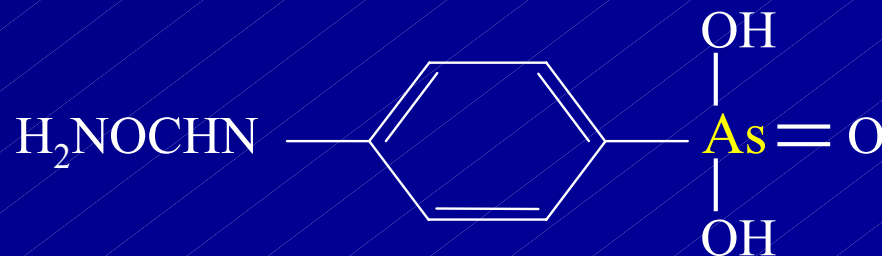
3-nitro-4-hydroxyphenylarsonic acid  
(3-NHPAA; Roxarsone)



4-nitrophenylarsonic acid  
4-NPAA



p-arsanilic acid  
p-ASA



p-ureidophenylarsonic acid  
p-UPAA





# Use of Roxarsone as Feed Additive for Broiler Chickens

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- According to Alpharma,<sup>37</sup> the manufacturer of the 3-Nitro version of roxarsone, “this compound is fed at concentrations between 22.7 and 45.4 grams per ton of feed to most broiler chickens raised in the U.S. as well as many other countries”
- Roxarsone is used to control coccidial intestinal parasites, thereby improving feeding efficiency (weight gain per unit of feed).



# Current Knowledge on the Metabolic Fate of Roxarsone Fed to Broiler Chickens

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- Very little roxarsone is retained in the chicken meat (FDA limit is 0.5 ppm arsenic in chicken muscle tissue).
- Most of the roxarsone appears to be excreted unchanged.<sup>38, 39</sup>
- The transformation product 3-amino-4-hydroxyphenylarsonic acid (3-AHPAA, Figure 2) has been detected in the urine of hens fed roxarsone.<sup>40</sup>



# Current Knowledge on Arsenic in Litter of Roxarsone-treated Broiler Chickens

---

- Litter (bedding material ❖ and excrement) of broilers fed roxarsone-treated feed contains 15-30 ppm arsenic.<sup>38</sup>
- Roxarsone and 4-hydroxyphenylarsonic acid (4-HPAA) have been identified as predominant forms of arsenic in non-composted litter.<sup>41</sup>
- Inorganic and methylated arsenic species were found in composted litter samples.<sup>41</sup>
- Possible pathways for the biotransformation of roxarsone are outline in Figure 2.

❖ The bedding material normally consists of sawdust and wood chips.

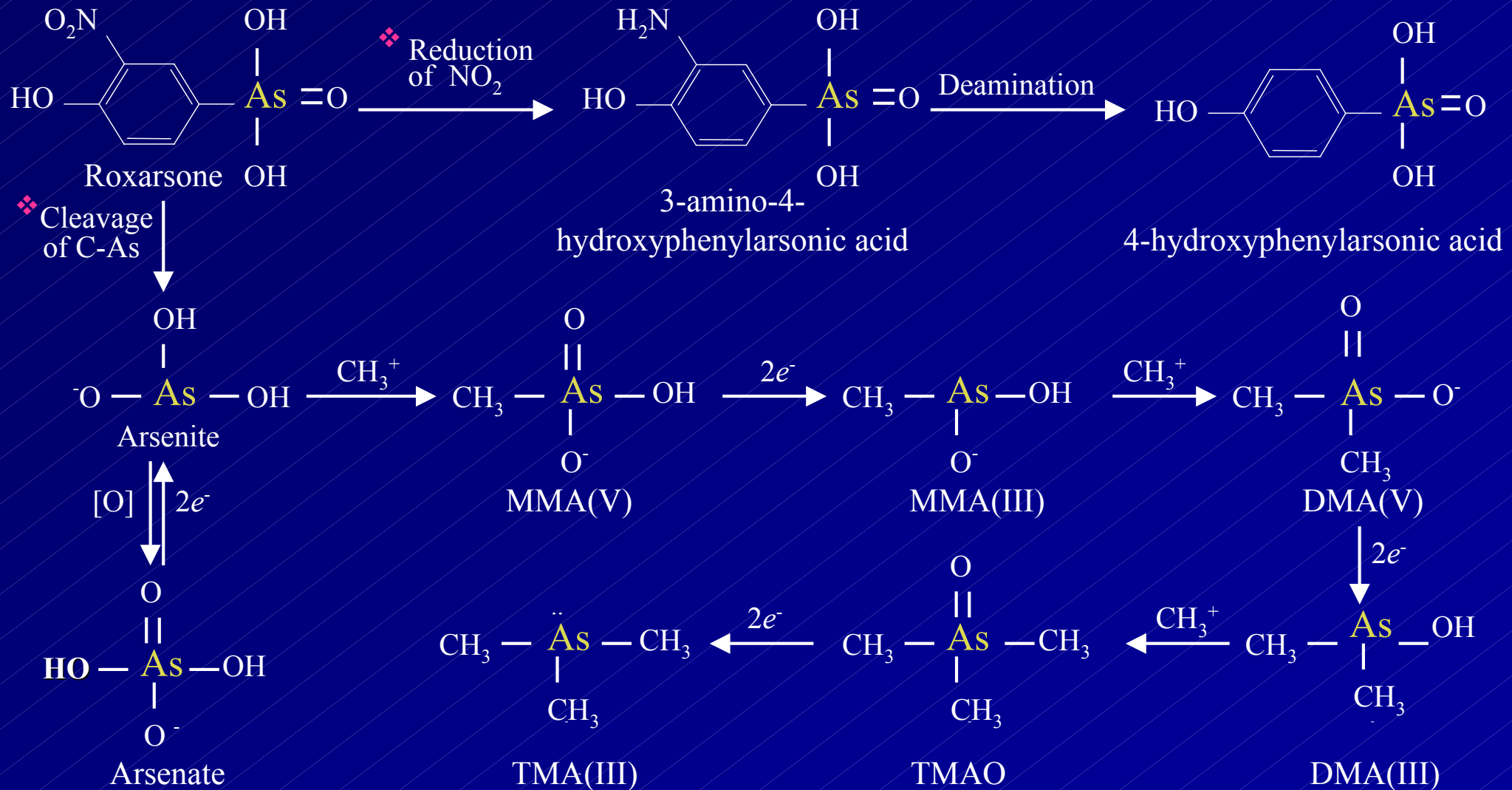


# Current Knowledge on Disposal Practice of Chicken Litter and Potential Arsenic Exposure

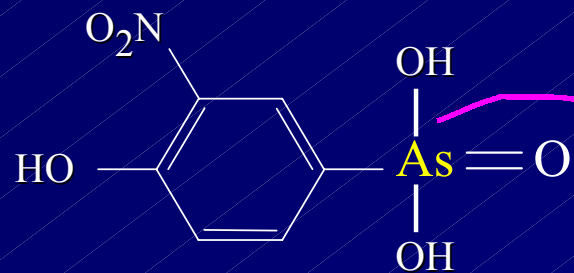
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- Poultry litter is generally used to fertilize agricultural fields near chicken houses.<sup>42, 43</sup>
- It is estimated that broiler chickens fed feed containing 45.4 g of roxarsone per ton for 42 days will excrete approximately 150 mg of roxarsone in total.<sup>44</sup>
- The Agricultural Marketing Service of the U.S. Department of Agriculture estimates that 8.15 billion broiler chickens were produced in the United States in 1999.
- If most of these chickens were fed feed containing roxarsone at the 45.4 g/ton rate, for 1999 alone, there was a potential for more than 300,000 kg of total arsenic to enter the environment through application of litter of roxarsone-treated broilers to agricultural land.
- The environmental impact may be substantial in geographic regions where the application of arsenic-contaminated litter is concentrated.
- Little is known about the environmental fate of roxarsone and transformation products identified in chicken litter.

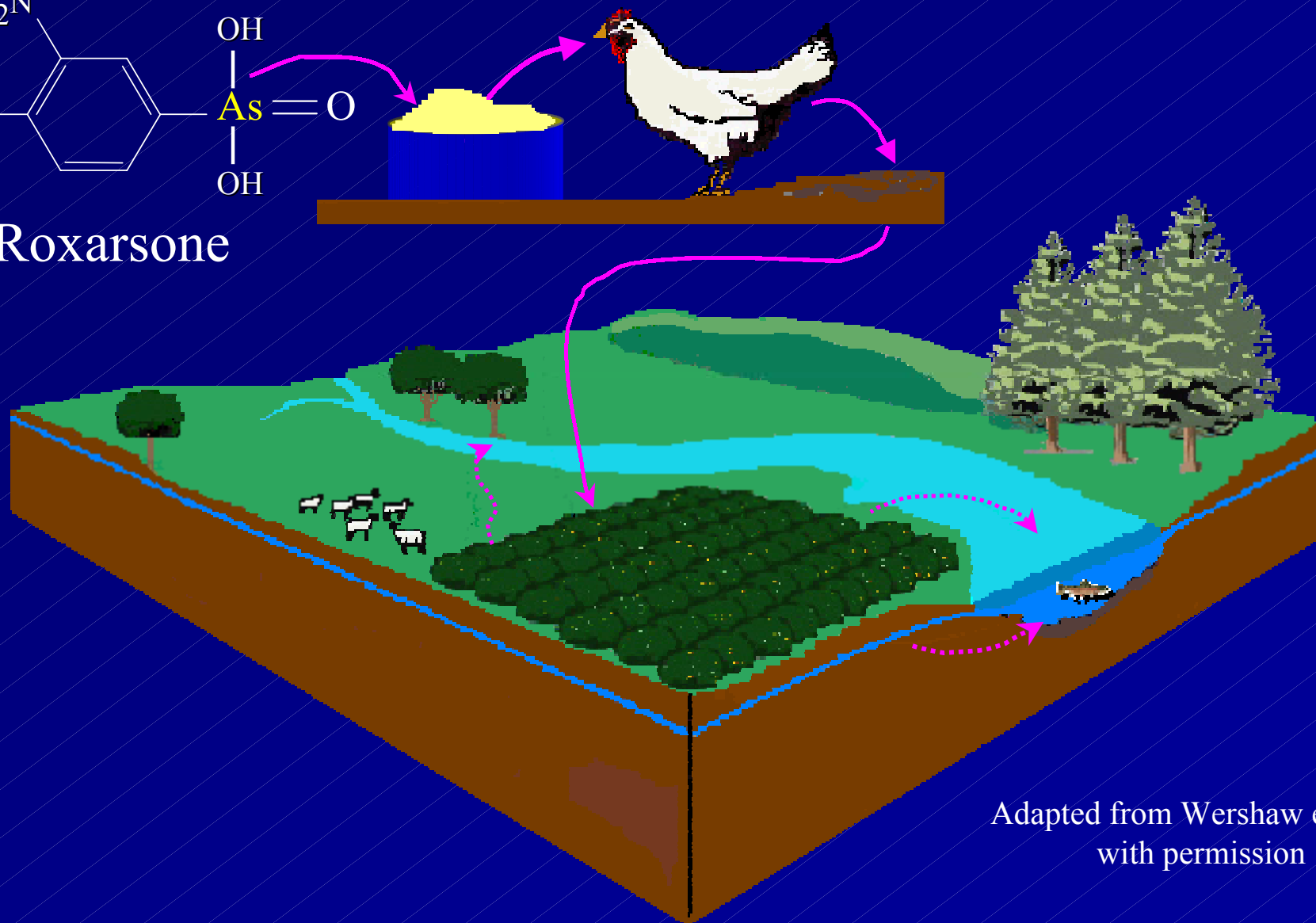
# Figure 2. Possible Pathways for the Biotransformation of Roxarsone



❖ Oxidative aromatic ring fission: If roxarsone were to undergo such a reaction sequence, arsonoalkyl acids would be produced. The arsonoalkyl acids could then undergo conversion to arsine as demonstrated by Challenger and Higginbottom.<sup>45</sup>



# Roxarsone



Adapted from Wershaw et al.<sup>46</sup>,  
with permission



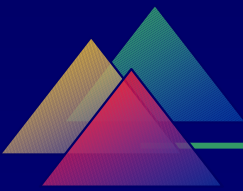


**Table1. Toxicity (LD<sub>50</sub>) of Potential Degradation Compounds of Roxarsone and Selected Organoarsenicals**

Arsenic Compounds	Subjects	Oral LD <sub>50</sub> (g/kg)
roxarsone	chickens	0.11 ❖
3-amino-4-hydroxyphenylarsonic acid		NR
4-hydroxyphenylarsonic acid		NR
dimethylarsinic acid	mice	1.2
methylarsonic acid	mice	1.8
arsenobetaine	mice	>10
arsenocholine	mice	6.5
trimethylarsine oxide	mice	10.6
tetramethylarsonium iodide	mice	0.89
arsenic trioxide	rats	0.034
potassium arsenite	rats	0.014
calcium arsenate	rats	0.020

NR: Not reported

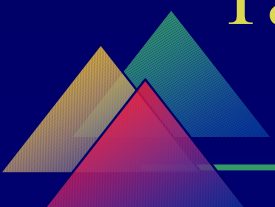
❖ A broiler chicken consumes approximately 3.5 mg of roxarsone per day for six weeks. This concentration of roxarsone is necessary to prevent coccidial infections and improve feed efficiency. However, as reported in this table, a 30-fold increase of this concentration when administered orally to experimental test chickens, resulted in the death of 50% of the exposed population.



# Development of Analytical Methods for the Measurement of Roxarsone and Potential Degradation Products

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- The analytical work included 11 arsenic species that were selected, based on the proposed transformation pathways described earlier, or because of their known usage as animal-feed additives. o-Arsanilic acid served as an internal standard.
- The separation principles selected for this study were ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) and capillary electrophoresis (CE).
  - The ion-pairing agents tested for the reversed-phase separations were mainly tetraalkylammonium salts.
- UV spectrophotometry and inductively coupled plasma mass spectrometry (ICPMS) were used to detect arsenic analytes.
  - Coupling of CE and microbore HPLC to ICPMS was effected via a high-efficiency nebulizer (HEN) or with a direct-injection high-efficiency nebulizer (DIHEN).

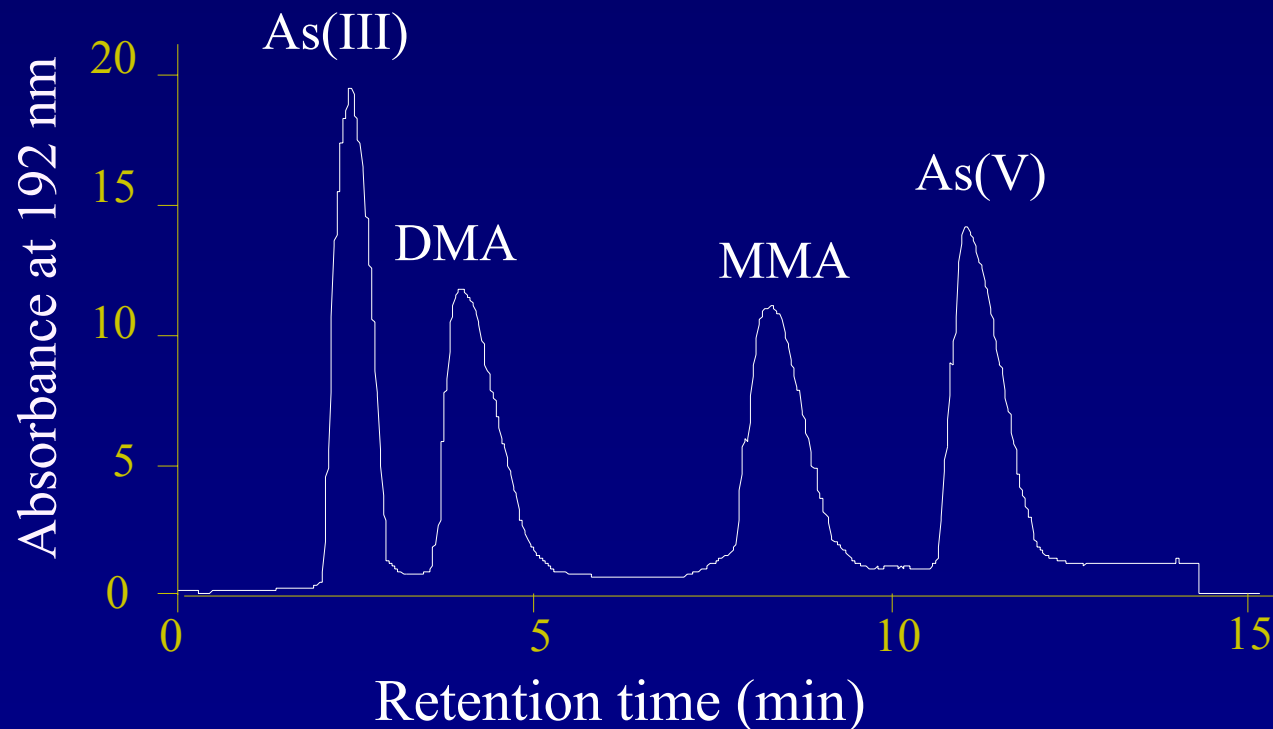


# Table 2. Names, Acronyms, Formulae of the Selected Arsenic Species.

Arsenic species	Acronyms	Formula
arsenous acid	As(III)	$\text{H}_3\text{AsO}_3$
arsenic acid	As(V)	$\text{H}_3\text{AsO}_4$
monomethylarsonic acid	MMA(V)	$\text{CH}_5\text{AsO}_3$
dimethylarsinic acid	DMA(V)	$\text{C}_2\text{H}_7\text{AsO}_2$
p-arsanilic acid	p-ASA	$\text{C}_6\text{H}_8\text{AsNO}_3$
o-arsanilic acid	o-ASA	$\text{C}_6\text{H}_8\text{AsNO}_3$
4-hydroxyphenylarsonic acid	4-HPAA	$\text{C}_6\text{H}_7\text{AsO}_4$
4-nitrophenylarsonic acid	4-NPAA	$\text{C}_6\text{H}_6\text{AsNO}_5$
3-nitro-4-hydroxyphenylarsonic acid ❖	3-NHPAA	$\text{C}_6\text{H}_6\text{AsNO}_6$
3-amino-4-hydroxyphenylarsonic acid	3-AHPAA	$\text{C}_6\text{H}_8\text{AsNO}_4$
p-ureidophenylarsonic acid	p-UPAA	$\text{C}_7\text{H}_9\text{AsN}_2\text{O}_4$

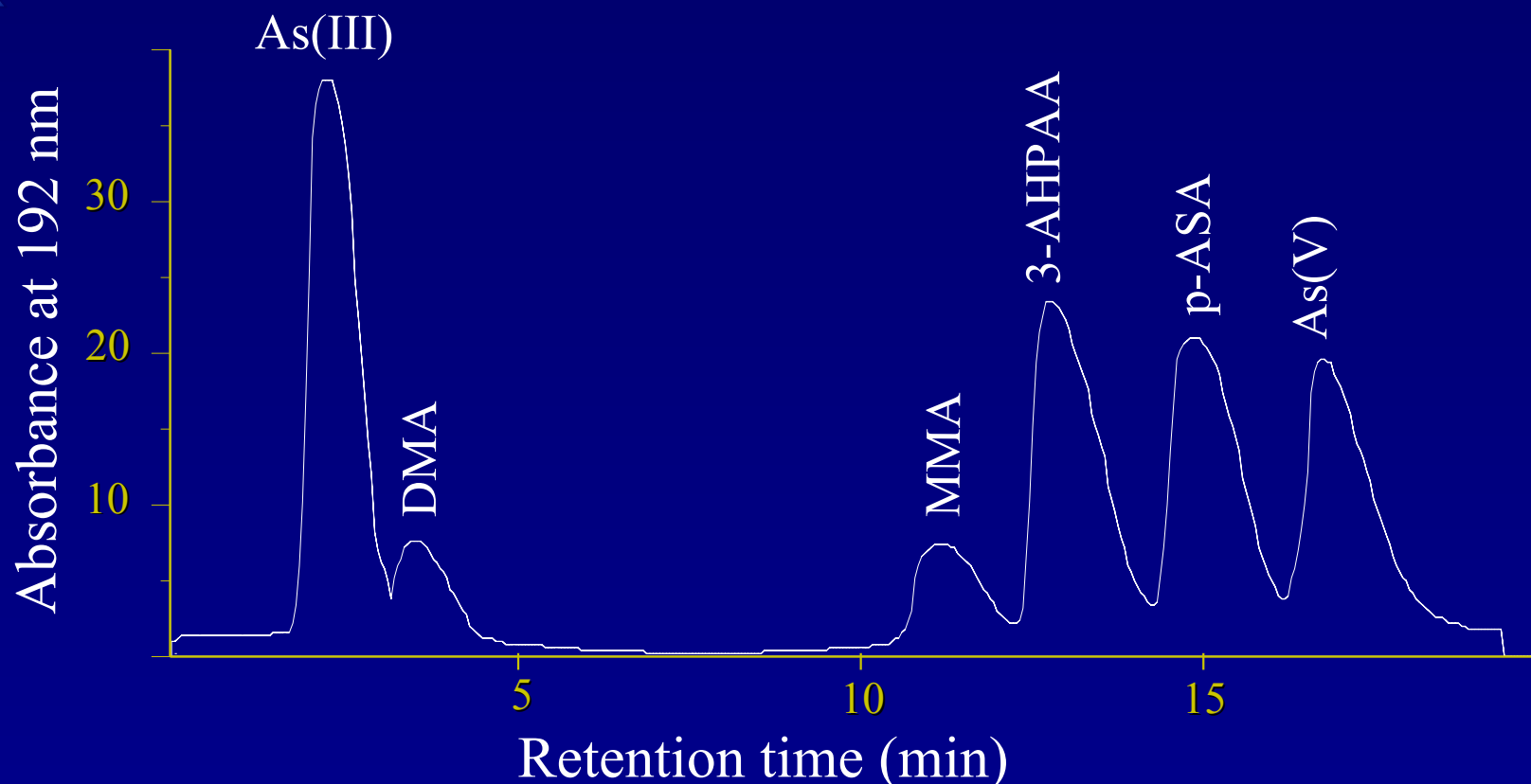
❖ Roxarsone

# Figure 3. IP-RP-HPLC-UV Chromatogram of Common Environmental Arsenic Species



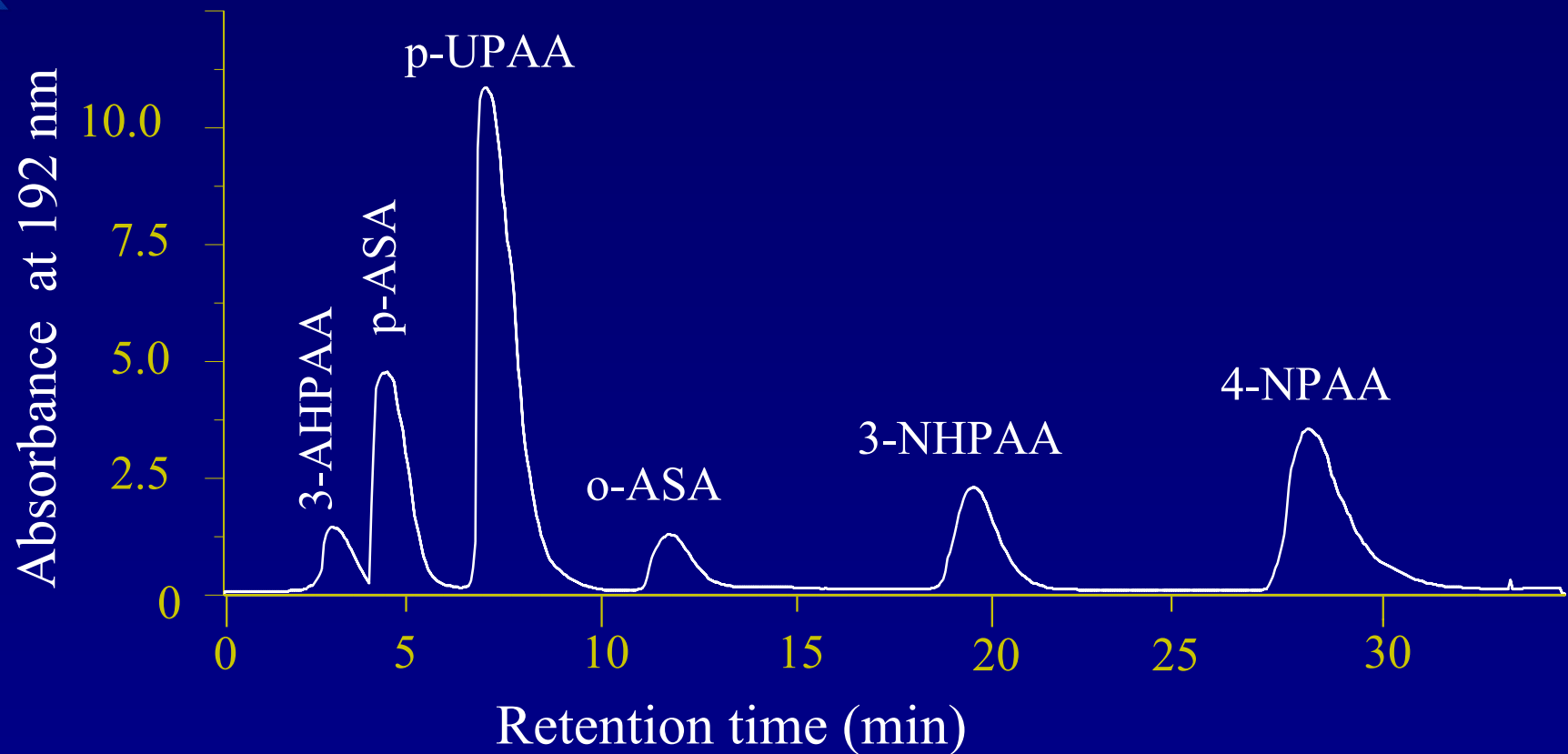
- Mobile phase composition: 1mM tetrapropylammonium hydroxide, 0.5% methanol, pH 4.0. The eluent was delivered to the C<sub>18</sub> column (2.1 mm i.d. x 250 mm, Supelco, Inc.) at a flow rate of 0.30 mL/min. The chromatographic separation was performed at room temperature.
  - Under the above chromatographic conditions, MMA and 3-AHPAA were only slightly separated. 4-HPAA eluted from the chromatographic column after 15 min. Roxarsone and the other phenylarsonic acids were highly retained.

## Figure 4. IP-RP-HPLC Separation of Common Environmental Arsenic Species and Two Phenylarsonic Acids



- HPLC conditions: 1 mM methyltributylammonium hydroxide, 0.5 % methanol, pH 4.0, delivered to the C<sub>18</sub> column at a flow rate of 0.30 mL/min.
  - MMA and 3-AHPAA are now resolved. 4-HPAA (not shown on this chromatogram) became more retained. Roxarsone was not eluted from the C<sub>18</sub> column even one hour later.

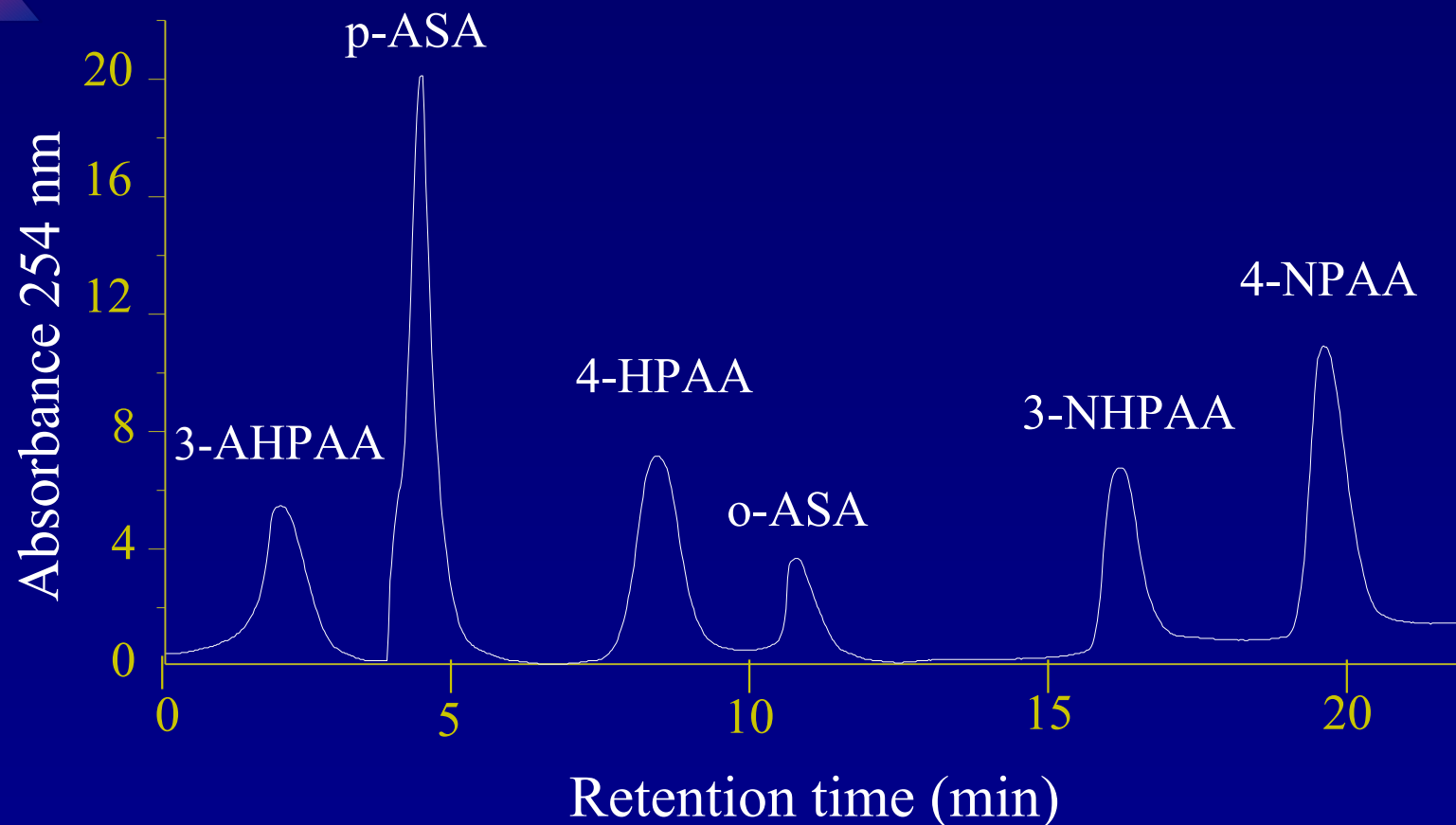
## Figure 5. IP-RP-HPLC Separation of Six Phenylarsonic Acid



- By changing the pH of the methyltributylammonium hydroxide solution to 2.5 and maintaining the other parameters constant, the four phenylarsonic acids animal-feed additives, and the reduction product of roxarsone eluted from the HPLC column within 30 minutes. It was also possible to separate the para- and ortho-isomers of arsanilic acid in the same chromatographic run. However under these conditions, As(III), As(V), DMA, and MMA were eluted in the void volume.

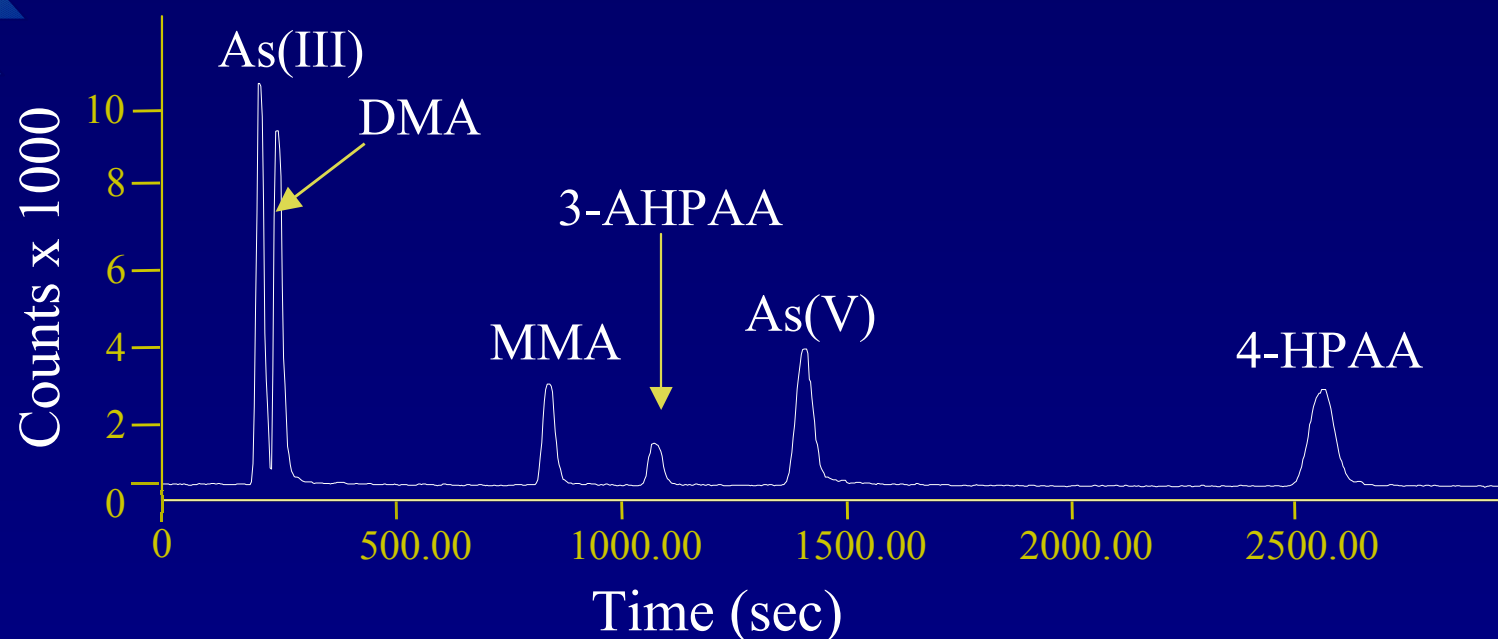


# Figure 6. IP-RP Chromatogram of Six Organoarsenic Compounds



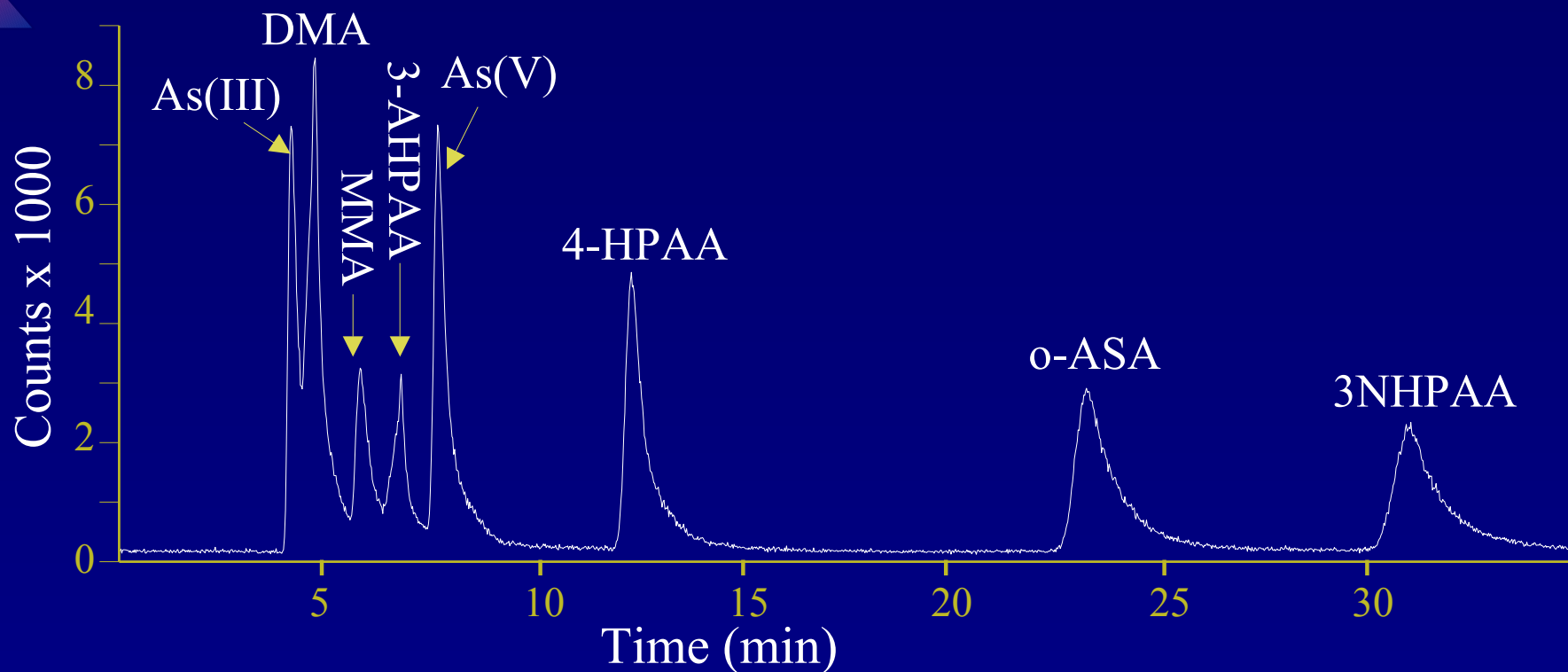
- Using the same chromatographic column, but this time with a 5 mM tetramethylammonium hydroxide solution, containing 3% methanol (pH 2.7), six phenylarsonic acids were efficiently separated. The increased concentration of ion-pairing agent and methanol in the mobile phase did not affect detector responses.

# Figure 7. HPLC-ICPMS Separation of Six Arsenic Compounds



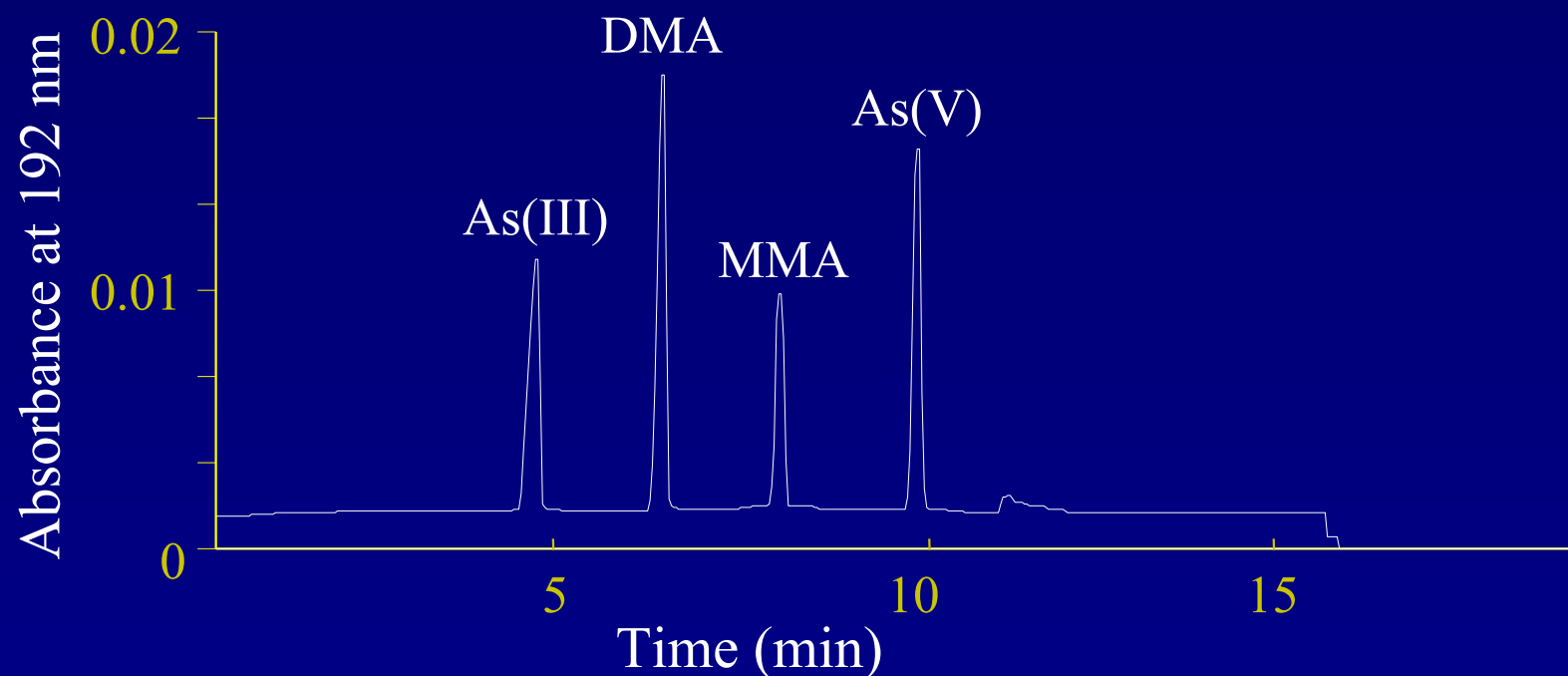
- Arsenite, arsenate, DMA, MMA, 3-AHPAA, and 4-HPAA were separated on a C<sub>18</sub> column (2.0 mm i.d. x 150 mm, Aqua, Phenomenex Inc.) which operated efficiently with 100% percent aqueous solution. Methyltributylammonium hydroxide was used as ion-pairing agent. Its concentration in the mobile phase was kept at 1mM and the pH of the solution was adjusted to 4.
  - The eluent was delivered to the chromatographic column at a flow rate of 100  $\mu$ L/min using a syringe pump. For improved nebulization efficiency at this low flow rate, the HEN was used to couple the HPLC system to the ICPMS.
  - Under the described chromatographic conditions, 4-HPAA eluted from the HPLC column 40 minutes later, but roxarsone remained strongly retained.

## Figure 8. $\mu$ HPLC-ICPMS Detection of Eight As species



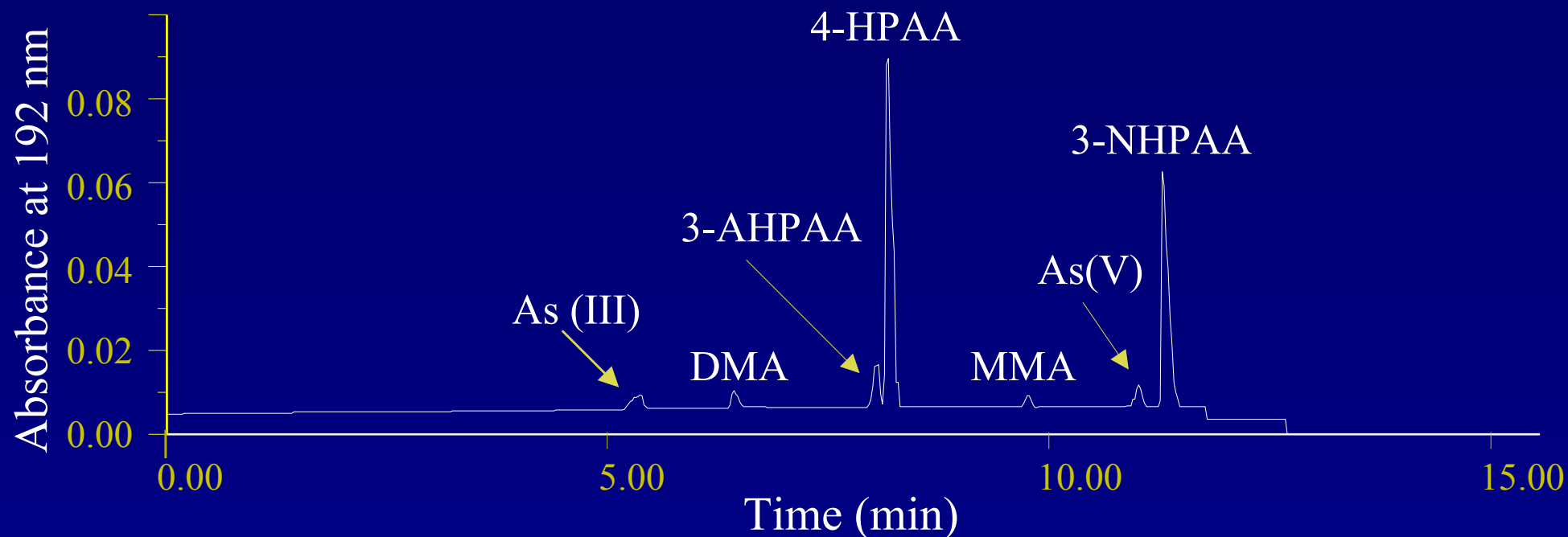
- When the Aqua C<sub>18</sub> column was used with a methanol-rich mobile phase containing methyl-tripropylammonium hydroxide as ion-pairing agent, the chromatographic retention time of 4-HPAA was reduced to ca. 14 minutes. For the first time, it was possible to separate roxarsone and two of its hypothesized organic transformation products, and the inorganic and methylated arsenic species in the same chromatographic run and under 30 minutes. The combination of methanol and methyl-tripropylammonium ion in the mobile phase was necessary for the successful separation of the selected arsenic compounds. o-Arsanilic acid served as an internal standard.

# Figure 9. Separation of Common Environmental Arsenic Species by Capillary Electrophoresis



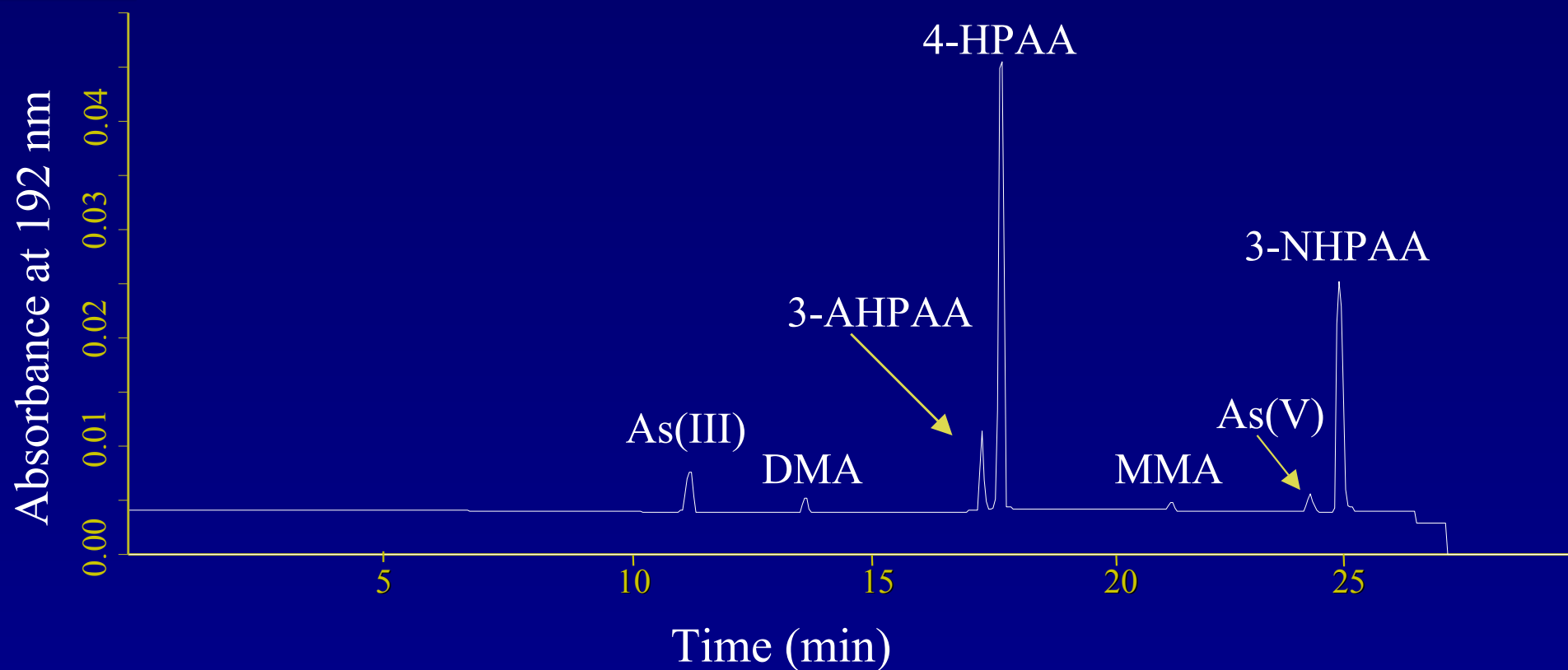
- The four commonly occurring inorganic and methylated arsenic species were separated to baseline using 15 mM phosphate buffer pH 6.6. The temperature was maintained at 25°C, and the electric field was adjusted to 15 kV. A fused silica capillary 57 cm x 75 µm i.d. was used to minimize run time.

## Figure 10. Separation of Seven Arsenic Species by Capillary Electrophoresis



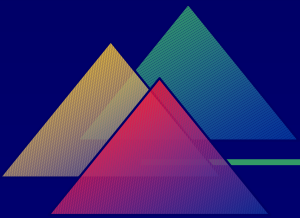
- This electropherogram illustrates the separation of roxarsone and its hypothesized degradation products.
- The arsenic compounds were separated using a fused silica capillary column (57 cm x 75  $\mu$ m i.d.) with 20 mM phosphate buffer pH 5.9 at 22  $^{\circ}$ C and 15 kV.
  - Under these conditions, roxarsone exited in less than 12 minutes. The reduction product of roxarsone (3-AHPAA) and 4-HPAA migrated closely to each other.

## Figure 11. Separation of Seven Arsenic Species by UV-Capillary Electrophoresis



- Using a longer-length capillary column (107 cm x 75  $\mu\text{m}$  i.d.) and increasing the applied voltage to 27 kV to maintain the same electrical field, while keeping the remaining parameters constant, a net improvement in the separation of 3-amino-4-hydroxyphenylarsonic acid and 4-hydroxyphenylarsonic acid was observed. However, the main purpose for using a longer capillary column was to facilitate coupling of the CE apparatus to the ICPMS in later experiments.

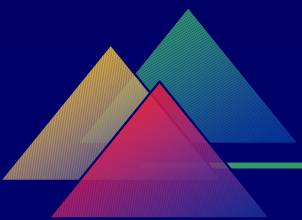




# Conclusions

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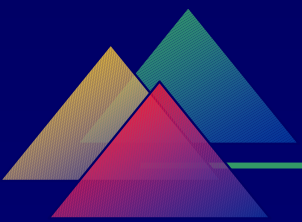
- Substituted phenylarsonic acids and common environmental arsenic species can be separated by IP-RP-HPLC and CE.
- ICPMS provides sensitive detection of separated arsenic species.
- Research on coupling CE and gradient HPLC with ICPMS is ongoing.



# References

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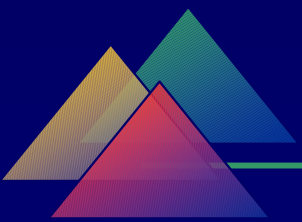
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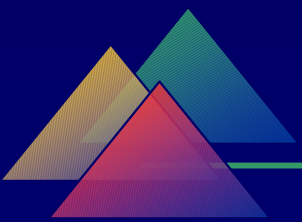
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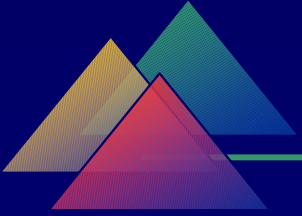


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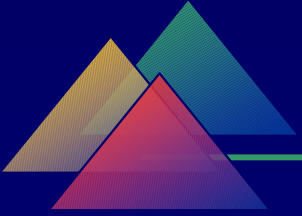




# Questions?

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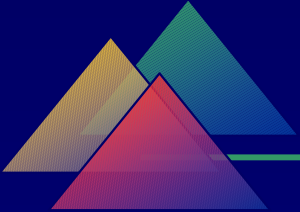
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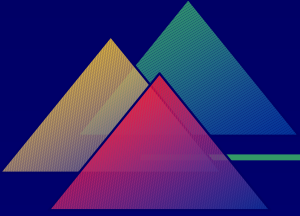
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